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Heavy Metals (Hg, Cd, Pb) Concentrations on *Mytilus galloprovincialis* (Lamarck, 1819) in Oran Coastal Waters (Western Algeria): New Evidences

J. Appl. Environ. Biol. Sci. 2015 5(6): 1-9. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Endah Angreni, Wahyono Hadi, Budisantoso Wirjodirdjo, Sarwoko Mangkoedihardjo

System Dynamic Modeling for Behaviour Pattern on Process and Operation of Water Treatment Plant

J. Appl. Environ. Biol. Sci. 2015 5(6): 10-15. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Muhammad Ishaqa, Qing Pinga, Chongguang Lia, Zahoor Ul Haq and Chen Tong

Food Safety and Trade Patterns: Case of Dairy in China

J. Appl. Environ. Biol. Sci. 2015 5(6): 16-24. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Ijaz Khalid and Zahir Shah

Beijing Reaction to Osama Operation inside Pakistan

J. Appl. Environ. Biol. Sci. 2015 5(6): 25-30. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Rasool Ghasemzadeh, Yunes Nikparast, Zahra Hejri, Esmaeil Koohestanian

Experimental and Kinetic Study of Zero-valent Iron Nanoparticles Performance for the Removal of Chromium from Oil Industry Waste water

J. Appl. Environ. Biol. Sci. 2015 5(6): 31-39. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Muhammad Anshari, Tri Martiana, Suhartono Taat Putra, L. Dyson

Ethnomedicine of Dayak Paramasan Ethnic in the Meratus Mountains (part-2): A Correlation of Spiritual Belief and Distress Level to Patient who had.....

J. Appl. Environ. Biol. Sci. 2015 5(6): 40-42. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Ghasemi, S., Mola, N., Hoseinpour, M., Hosseini-Tayefeh, F., Najji, A., Pakravan, J.

Coral Diversity of Hengam Island, Persian Gulf, Iran

J. Appl. Environ. Biol. Sci. 2015 5(6): 43-52. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Bushra Hamid, Inayat ur-Rehman, Abdul Rauf, Tamim Ahmed Khan

Using Smote for Convalescing Software Defect Prediction

J. Appl. Environ. Biol. Sci. 2015 5(6): 53-59. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Muhammad Rendana, Sahibin Abdul Rahim, Tukimat Lihan, Wan Mohd Razi Idris, Zulfahmi Ali Rahman

A Review of Methods for Detecting Nutrient Stress of Oil Palm in Malaysia

J. Appl. Environ. Biol. Sci. 2015 5(6): 60-64. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Jalalabadi. Leila, Ghasemi. Fourogh, Sadryfard. Afsane, Kiani Ghale No. Zahra

Social – Economical Evaluation of Sustainable Urban Development Using Regional Planning Models (Case Study: Bam City-Iran)

J. Appl. Environ. Biol. Sci. 2015 5(6): 65-73. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Mohsen Zargham, Seyed Mohammad Shobeiri, Mohammad Reza Sarmadi, Mohammad Hassan Seif

Causal Model to Explain the Students` Willingness to Use Cell Phone in Environmental Education

J. Appl. Environ. Biol. Sci. 2015 5(6): 74-82. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Nimatuzahroh, Rima Nurmalsari, Rozy Ayu Silvia, Tri Nurhariyati, Tini Surtiningsih

Effectiveness in Enhancing Oil Recovery through Combination of Biosurfactant and Lipases Bacteria

J. Appl. Environ. Biol. Sci. 2015 5(6): 83-87. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Farid Golzardi, Yazdan Vaziritabar, Yavar Vaziritabar, Kamal Sadat Asilan, Mohamad Hasan Jafari Sayadi, Shabnam Sarvaramini

Effect of Solarization and Polyethylene Thickness Cover Type on Weeds Seed Bank and Soil Properties

J. Appl. Environ. Biol. Sci. 2015 5(6): 88-95. [\[Abstract\]](#) [\[Full Text PDF\]](#)

M. Mohseni, M. R. Ardekani, S. Vazan, F. Paknezhad

The Effect of Mycorrhiza Inoculation and Azotobacter Strains on the Characteristics of the Root of Cuminum Cyminum in Agroecosystem without.....

J. Appl. Environ. Biol. Sci. 2015 5(6): 96-104. [[Abstract](#)] [[Full Text PDF](#)]

Nurul Jadid, Tutik Nurhidayati, Priyono

In Vitro Clonal Propagation of Vanilla planifolia Andrews Using Microshoot-derived Node Explants

J. Appl. Environ. Biol. Sci. 2015 5(6): 105-110. [[Abstract](#)] [[Full Text PDF](#)]

Nasrin Ziadi, Seyed Rasul Emadi

Examine the Role of Educational Painting Thematic and Complementary Methods in Learning and Retention of the Words and Phrases in English

J. Appl. Environ. Biol. Sci. 2015 5(6): 111-122. [[Abstract](#)] [[Full Text PDF](#)]

Fariba Korani, Hamed Ghaderzadeh, Mehdi Korani

Economic Study of BMS Application in Building and Affective Factors in Adaption

J. Appl. Environ. Biol. Sci. 2015 5(6): 123-129. [[Abstract](#)] [[Full Text PDF](#)]

Zahir Shah and Ijaz Khalid

Beijing's Muteness on Drone Attacks inside Pakistan

J. Appl. Environ. Biol. Sci. 2015 5(6): 130-135. [[Abstract](#)] [[Full Text PDF](#)]

Seyed Mohammad Hossein Nasehi, Iraj Jalali, Ebrahim Khorasani Parizi

Analysis of Internet Role on Development of Women Social Rights in Iran Society

J. Appl. Environ. Biol. Sci. 2015 5(6): 136-149. [[Abstract](#)] [[Full Text PDF](#)]

Seyed Alireza Seyed Salehi, Haleh Samadi

Investigation of Challenges Ahead of Globalization of Iranian Small and Medium-Sized Enterprises Process via Export

J. Appl. Environ. Biol. Sci. 2015 5(6): 150-157. [[Abstract](#)] [[Full Text PDF](#)]

Saeid Kheradmandy, Mohammad Husain Sadeghi

Presumption of Innocence Juridical-Legal Checking

J. Appl. Environ. Biol. Sci. 2015 5(6): 158-164. [[Abstract](#)] [[Full Text PDF](#)]

Zahra Tavana, Rasool Roshan Chasli, Leila Heidari Nasab, Karim Golmohammadi

Evaluate the Effectiveness of School-Based Cognitive-Behavioral Group Therapy on Reduction of Anxiety in Children

J. Appl. Environ. Biol. Sci. 2015 5(6): 165-170. [[Abstract](#)] [[Full Text PDF](#)]

Reza Gholami Majin, Maryam Eslampanah, Babak Jamshidinavid

The Role of Knowledge Management Enabler on Performance Kermanshah Province Maskan Bank

J. Appl. Environ. Biol. Sci. 2015 5(6): 171-177. [[Abstract](#)] [[Full Text PDF](#)]

Hassan Rezaee Haftador

Examination of the Arrangement of the Quran

J. Appl. Environ. Biol. Sci. 2015 5(6): 178-185. [[Abstract](#)] [[Full Text PDF](#)]

Rohollah Karami, Hamid Reza Mehrabi, Ali Ariapoor

Factors Impact of Organic Matter, NPK, EC and pH of Soil on Species Diversity in the Watershed of Miandar Qarootag – Gilangharb

J. Appl. Environ. Biol. Sci. 2015 5(6): 186-190. [[Abstract](#)] [[Full Text PDF](#)]

Saeid Kheradmandy, Shahram Lorestani

Criminal Liability Arising from Sporting Events, to Look at the Principles of Jurisprudence in Iran

J. Appl. Environ. Biol. Sci. 2015 5(6): 191-195. [[Abstract](#)] [[Full Text PDF](#)]

Aman-Mohammad Amanjani, Parvaneh Espoo, Ramzan-Ali Zanganeh

Codification of Strategic Planning in Healthcare Field of Social Welfare Organization

J. Appl. Environ. Biol. Sci. 2015 5(6): 196-205. [[Abstract](#)] [[Full Text PDF](#)]

Zahir Shah and Ijaz Khalid

Pakistan-Iran Relations in the Changing Global Scenario Post 9/11

J. Appl. Environ. Biol. Sci. 2015 5(6): 206-212. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Morteza Esmaeeli, Alireza Moradi

The Impact of Supply Chain Integration on Customer Satisfaction and Financial Performance of Manufacturing Firms Kermanshah City

J. Appl. Environ. Biol. Sci. 2015 5(6): 213-222. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Fatemeh Jalilian, Omid Jalilian, Seyed Reza Hassani

A Study of the Impact of Competitive Intelligence on Financial and Non-Financial Performance of Saman Gharb Cement Industries Company

J. Appl. Environ. Biol. Sci. 2015 5(6): 223-228. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Saeid Kheradmandy, Majid Rashidian

The Presumption of Innocence

J. Appl. Environ. Biol. Sci. 2015 5(6): 229-233. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Parnia Dana, Mehrdad Ghanbari

The Effect of Earnings Response Coefficient in the Abnormal Efficiency

J. Appl. Environ. Biol. Sci. 2015 5(6): 234-240. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Shoeleh Movahhed, Mansoureh Aligholi

Investigation of Relationship between Knowledge Management Maturity Level and Customer Relationship Management in Karafarin Bank in Tehran City

J. Appl. Environ. Biol. Sci. 2015 5(6): 241-246. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Fateme Baegi Mirazizi, Alimohammad Mokarrami

Comparative Study of the Nature and Legal Effects of Apartheid in Islam and International Law

J. Appl. Environ. Biol. Sci. 2015 5(6): 247-251. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Mohammad Motalebi Nejad, Mansoureh Aligholi

Relationship between Transformational Leadership and Organizational Forgetting and its Impact on Competitiveness of Organizations (Case Study:.....)

J. Appl. Environ. Biol. Sci. 2015 5(6): 252-257. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Fatemeh Ferdosian, Ramin Zafarmehrabian, Hamid Reza Pordeli

The Green Synthesis of N-Succinyl Chitosan/Ag Nanocomposites via Ultrasonic Process and Investigation of their Antibacterial Properties

J. Appl. Environ. Biol. Sci. 2015 5(6): 258-263. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Shahnaz Jamalzad Azad, Mousa Kafi

Investigation of the Impact of Group Narrative-Therapy on Increasing Old People's Happiness in Rasht City Nursery Homes

J. Appl. Environ. Biol. Sci. 2015 5(6): 264-267. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Reza Gholamnia, Ahmad Alibabaei, Mousa Jabbari, Hedayat Allah Kalantari

Fuzzy Risk Assessment of Fire and Explosion in the Crude Oil Storage Tanks by Fuzzy Hierarchical Analysis

J. Appl. Environ. Biol. Sci. 2015 5(6): 268-272. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Mohammad Reza Maleknejad, Massoud Amoopour, Effat Abdi

Content Analysis of Biology Book of Grade Three from Students of Public and Private High Schools Viewpoint in Rasht City during 2014-2015

J. Appl. Environ. Biol. Sci. 2015 5(6): 273-277. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Zohreh Feiz Abadi, Mania Salehi Far, Mohammad Reza Ishaghi

Investigating the Qualitative Characteristics of Toast Bread Obtained from Par-Baked Paste Kept in the Fridge and the Freezing Condition over Zero Degr

J. Appl. Environ. Biol. Sci. 2015 5(6): 278-286. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Shokoufeh Modanloo, Leila Dehghankar, Mitra Zolfaghari, Arezoo Mohammadkhani Ghiyasvand, Akbar Babaei Heydarabadi, Seyyed Hannan Kashfi

The Effects of Training and Follow-up via Text Messaging on Weight Control in Hemodialysis Patients

J. Appl. Environ. Biol. Sci. 2015 5(6): 287-292. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Asiyeh Norouzi, Hamid Samsam

An Analysis of Malamati and Qalandari Themes in Sana'll's and Hafez's Poems

J. Appl. Environ. Biol. Sci. 2015 5(6): 293-298. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Niloofer Emankhan, Hamideh Sohrabian Azar Dizag

Investigating the Effect of the Ethical Behavior of the Staff on the Satisfaction and Loyalty of the Bank Services Recipients (A Case Study of Bank Sader)

J. Appl. Environ. Biol. Sci. 2015 5(6): 299-311. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Niloofer Emankhan, Maryam Karimi

The Effect of Export Market Orientation on the Automobile Spare Parts Export Performances

J. Appl. Environ. Biol. Sci. 2015 5(6): 312-323. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Niloofer Emankhan, Seyed Ali Derogar Kasmaei

A Study of the Attractiveness Factors of an Organization's Brand from the Viewpoints of the Staff Based on "Age, Sex, Experience, and Employment

J. Appl. Environ. Biol. Sci. 2015 5(6): 324-333. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Samira Soheili Rad

Critical Success Factors (CSFs) in Strategic Planning for Information Systems

J. Appl. Environ. Biol. Sci. 2015 5(6): 334-339. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Hormoz Asadi Koohbad, Mohsen Poorjam

The Civil Liability of Inspectors in EPC Contracts

J. Appl. Environ. Biol. Sci. 2015 5(6): 340-351. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Fatemeh Bagheri, Arian Satei

Effect of Ascorbate and Methylamine Treatment on Glycinebetaine, Sugar, and Proline Contents of Soybean (*Glycine max L.*) Seeds under Polyethylene..

J. Appl. Environ. Biol. Sci. 2015 5(6): 352-357. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Somayeh Yazdani

Characterizations of Quasiconvex Functions and Pseudomonotonicity of Subdifferentials

J. Appl. Environ. Biol. Sci. 2015 5(6): 358-364. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Majid Golparvar, Omid Nouri

The Influence of Sunni Political Fiqh on the Recent Egyptian Developments

J. Appl. Environ. Biol. Sci. 2015 5(6): 365-373. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Abdolreza Yazdani, Sara Golcheh

Hydrodynamic Investigation of Bubble Formation in Non-Newtonian Fluids in Waste Water Filtration Aeration Tanks Using CFD

J. Appl. Environ. Biol. Sci. 2015 5(6): 374-384. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Mohammad Reza Kia, Mina Jamshidi Avanaki

Investigation of the Influence of Bank Features and Banking Industry Features on Profitability of Banks

J. Appl. Environ. Biol. Sci. 2015 5(6): 385-391. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Atefe Movaghar, Hossein Akbari Amarghan

Effectiveness of Hope Therapy on Resilience of Adolescents in Group

J. Appl. Environ. Biol. Sci. 2015 5(6): 392-397. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Zahra Moussavi Moghadam, Loghman Ahmadipour, Ghobad Yosefi, Hasan Binandeh, Abbas Ranjbari

Effectiveness of Resiliency Skills Education on Quality of Life and Reduce Couples Conflicts

J. Appl. Environ. Biol. Sci. 2015 5(6): 398-403. [\[Abstract\]](#) [\[Full Text PDF\]](#)

Effectiveness in Enhancing Oil Recovery through Combination of Biosurfactant and Lipases Bacteria

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Received: February 2, 2015

Accepted: April 10, 2015

ABSTRACT

Oil pollution accidents recently became phenomenon and caused accidental contamination of ecosystem. It originated from leaking pipes, transportation accidents, and damage oil storage tanks that contaminated both soil and groundwater. One of the solution was oil recovery process using sand pack column with combination of biosurfactant (*Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61 and *Acinetobacter* sp. P2(1)) and lipases (*Actinobacillus* sp., *Micrococcus* sp. L II). Sand-pack column model designed to simulate oil recovery operations and evaluate the mobilization of residual oil by combined biosurfactants and lipases. This study was an experimental study through four replications. Treatment were consists of eight combinations; *Acinetobacter* sp. P2 (1) - *Actinobacillus* sp., *Bacillus subtilis* 3Kp - *Actinobacillus* sp., *Pseudomonas putida* T1-8 - *Actinobacillus* sp., *Micrococcus* sp. L II 61 - *Actinobacillus* sp., *Acinetobacter* sp. P2 (1) - *Micrococcus* sp. L II 61, *Bacillus subtilis* 3Kp - *Micrococcus* sp. L II 61, *Pseudomonas putida* T1-8 - *Micrococcus* sp. L II 61 and biosurfactant and lipase *Micrococcus* sp. L II 61 with positive control synthetic surfactant (Tween-20). The results of treatment were extracted using n-hexane. Effectiveness of oil recovery by biosurfactants and lipases combination analyzed using One-way ANOVA test. These eight combinations effectively mobilize of entrapped oil as indicated by high percentage of oil recovered compared to the synthetic surfactants (Tween-20). Thus, the eight types of combination were capable to replace synthetic surfactants in oil recovery processes using sand pack column.

KEYWORDS— Oil recovery, Bacteria, Biosurfactants, Lipases, Sand pack column

INTRODUCTION

Oil existence in waters and land was considered to be undesirable, both because of its quantity and in the wrong place [1]. Product of oil processing industry had potentially polluted the environment and caused damage or disturb the living things. Waste produced by oil processing was in form of hydrocarbon compound. Hydrocarbon waste formed from leaks on transportation pipe and raw oil storage tank damage, both were capable of causing pollution [2].

The effective and efficient method to handle oil waste pollution was to elevate oil recovery, by surfactant administration. Surfactant (*surface active agent*) was amphipathic molecule consisted of hydrophobic and hydrophilic groups. Chemical surfactant usage possibly caused environmental problem of its resistant properties and high toxicity when it accumulated on natural ecosystem [3], so various environmental-friendly surfactant as alternative means started to be developed. One of it was by using microorganism which known as biosurfactant. Biosurfactant capable of declining surface tension, increasing solubility of hydrophobic compound contained in oil, and extending facilitation for microbe on hydrocarbon degradation [4].

Lipases were one material also able to elevate oil recovery. Lipases addition could increase oil recovery effectiveness because it properties in degrading oil hydrocarbon compound. In addition, hydrocarbonoclastic microbe also produced certain compound namely biosurfactant [5]. Because of that, formula addition of potential microbe consortium from lipase-producing bacterial group was necessary on furthering oil recovery effectiveness [6].

Oil recovery effectiveness affected by biosurfactant and lipase types used to dissolve the oil. Effectiveness in oil waste recovery process using biosurfactant and lipase rarely applied before. For that, it required to test oil waste mobility using sand pack column. Sand pack column method was one of many kind method used to determine oil recovery capability.

Biosurfactant-producing bacterial used in this study were *Acinetobacter* sp. P2(1), *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, and *Micrococcus* sp. L II 61, while lipase-producing bacterial that used were *Actinobacillus* sp. and *Micrococcus* sp. L II 61. Treatments consisted of 8 combination; *Acinetobacter* sp. P2(1) - *Actinobacillus* sp., *Bacillus subtilis* 3Kp - *Actinobacillus* sp., *Pseudomonas putida* T1-8 - *Actinobacillus* sp., *Micrococcus* sp. L II 61 - *Actinobacillus* sp., *Acinetobacter* sp. P2(1) - *Micrococcus* sp. L II 61, *Bacillus subtilis* 3Kp - *Micrococcus* sp. L II 61, *Pseudomonas putida* T1-8 - *Micrococcus* sp. L II 61 and *Micrococcus* sp. L II 61 biosurfactant and lipase, with synthetic surfactant (Tween-20) as positive control.

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METHODS

This study conducted in Microbiology Laboratory of Biology Department, Faculty of Science and Technology, Airlangga University at January – May 2014.

A. Lipase and Biosurfactant Production Medium Preparation

Lipase production medium used is 93 ml Nutrient Broth mixed with 2 ml cooking oil, sterilized in 500 ml bottle. Biosurfactant production medium used Synthetic Mineral Water (SMW) Medium, made by dissolving 3 g (NH₄)₂SO₄, 0.2 g MgSO₄·7H₂O, 10 g NaCl, 0.01 g CaCl₂, 0.001 g MnSO₄·H₂O, 0.001 g H₃BO₃, 0.001 g ZnSO₄·7H₂O, 0.001 g CuSO₄·5H₂O, 0.005 g CoCl₂·6H₂O, and 0.001 g Na₂MoO₄·2H₂O into 980 mL aquadest and 20 mL diluted molasse in aquadest (1:1). The solution homogenized using magnetic stirrer and its pH neutralized by adding 10% NaOH or 5% HCl. Solution then put into 500 mL tube. Sterilized SMW and molasse medium is macronutrient solution. While micronutrient solution made from 1 g KH₂PO₄, 1 g K₂HPO₄ and 1 g FeSO₄·7H₂O in 50 mL aquadest stock, which then sterilized. Both nutrient solutions mixed in aseptic condition.

B. Biosurfactant Supernatant and Lipases Characteristic Test

Bacterial cells in culture incubated for 4 days separated from medium containing biosurfactant with 9000 rpm, 4°C centrifuge for 15 minutes [7]. Before recovering oil, potential and characteristic of biosurfactant bacterial supernatant was tested, because each bacterial have different ability on oil recovery, which would be affected solubility percentage. The abilities of supernatant acquired tested by measuring surface tension and emulsification activity.

Lipase characteristic tested by calculated lipolytic activity if crude enzyme, measured quantitatively using modified [8] method. Lipolytic activity was determined using spectrophotometric method in *p*-nitrofenil palmitat (*p*-NPP) substrate.

C. Oil Recovery Test using Sand Pack Column Method

In mobilization test using sand pack column, initially sand filtered using size 40 mess, then it washed with acids (5% HCl) one times and shaken down to homogenized it. Then it was rinsed using aquadest for 3 times, dried by putting it on the oven of 50°C temperature, and weighted for 30 g. Sand mixed with 10 ml crude oil to saturate it. Sand mixture put into sand pack column, which beneath covered with milipore filter membrane (Whatman No.1). Biosurfactant and lipase (1:1) formulated solution of 10 ml added to it. Negative control used was aquadest and positive control used was Tween-20 synthetic surfactant. Formulated solution of biosurfactant and lipase flooded in glass tube was left in for 12-24 hours. Dissolved oil measured by extracting disintegrating oil from sand pack column using gravimetric method. N-hexane solvent added to the resulting solution, the solvent evaporated for ± 20 minutes in n-hexane boiling point of 60-70° C. Resulting oil of evaporation weighted.

RESULTS AND DISCUSSION

A. Supernatant characteristic of Biosurfactant and Lipase

1) Surface Tension

Table 1 showed that *Bacillus subtilis* 3Kp bacteria was able to decline surface tension as much as 9.14 dyne/cm. According to [9], surface tension decreased up to ≥10 dyne caused by production factor, of not optimum *Bacillus subtilis* 3Kp incubation time. *Bacillus subtilis* was surfactant producing microbe from subtilicine type.

Acinetobacter sp. P2(1) and *Pseudomonas putida* T1(8) bacteria were able to decrease surface tension by 16.89 dyne/cm and 10,56 dyne/cm. *Acinetobacter sp.* P2(1) and *Pseudomonas putida* T1(8) biosurfactant bacteria were able to descend surface tension as much as ≥10 dyne/cm. Both product of biosurfactant had small molecule weight, it indicated by high amount of surface tension decline [10].

Micrococcus sp. L II 61 reduced surface tension up to 17.82 dyne/cm. These results informed that *Micrococcus sp.* L II 61 also capable of declining surface tension by ≥10 dyne/cm. *Micrococcus sp.* L II 61 had the highest result in reducing surface tension.

Table 1. Surface Tension of 4 Types Supernatant Containing Biosurfactant

Treatment	Surface Tension (dyne/cm)	Surface Tension Reduction (dyne/ml)
Molasse without microbe	59.70 ± 1.35	-
Molasse + <i>Bacillus subtilis</i> 3Kp	50.56 ± 0.64	9.14
Molasse + <i>Acinetobacter sp.</i> P2(1)	42.81 ± 1.55	16.89
Molasse + <i>Pseudomonas putida</i> T1(8)	49.14 ± 1.85	10.56
Molasse + <i>Micrococcus sp.</i> L II 61	41.88 ± 1.60	17.82

2)

3) Emulsification Activity

Emulsification activity (EA) test also important in representing biosurfactant production by *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61 and *Acinetobacter* sp. P2(1). Emulsion stability (%) was show the strength of biosurfactant produced by microbes in emulsifying hydrocarbon [11].

Table 2 shows that *Bacillus subtilis* 3Kp able to emulsify oil as much as 18.85% with low stability percentage of 4.05%, while *Acinetobacter* sp. P2(1) and *Micrococcus* sp. L II 61 able to emulsify raw oil by 24.94% and 63.97%, both condition were relatively stable after 24 hours, by 20.88% and 61,50% with low stability percentage of 4.48% and 2.47%. Biosurfactant of *Pseudomonas putida* T1(8) was able to emulsify raw oil by 40.64% with high stability percentage of 34.4%. From this results, it concluded that the four bacterias capable of producing bioemulsifiers compound with low stability percentage, except *Pseudomonas putida* T1(8). But the highest emulsification activity acquired from *Micrococcus* sp. L II 61.

Table 2. Emulsification Activity for 1 hour and 24 hours (%) of supernatant containing biosurfactant towards crude oil

Treatment	Average Emulsification Activity (%)		Reduction of Emulsification Activity (%)
	1 hour	24 hours	
Tween-20 control	47.14 ± 13.9	46.39 ± 13.04	0.75
<i>Bacillus subtilis</i> 3Kp	18.85 ± 2.90	14.38 ± 4.05	4.05
<i>Acinetobacter</i> sp. P2(1)	24.94 ± 2.92	20.88 ± 3.47	4.48
<i>Pseudomonas putida</i> T1(8)	40.64 ± 10.38	6.25 ± 0.58	34.4
<i>Micrococcus</i> sp. L II 61	63.97 ± 5.31	61.50 ± 2.28	2.47

4) Lipolytic Activity Test

Lipase catalyzed triglycerides that hydrolyzed into diglycerides, monoglycerides, glycerols, and fatty acids. Triglycerides were split into fatty acid and glycerol by lipase is called lipolytic activity. Lipolytic activity of lipase crude enzyme measured quantitatively using *p*-nitrofenil palmitat (*p*-NPP) as testing substrate.

Table 3 showed that *Micrococcus* sp. L II 61 had the higher activity amount by 11.137 (U/ml), while *Actinobacillus* sp. had the lower activity average of 7.553 (U/ml). Both bacterias have lipolytic ability and can be used in oil recovery test.

Table 3. Lipolytic activity and dry weight of Lipase crude enzyme raw product

Bacterial Lipase	Average Activity (U/ml)	Dry Weight (g)
<i>Micrococcus</i> sp. L II 61	11.137 ± 0.566	0.762
<i>Actinobacillus</i> sp.	7.553 ± 2.99	0.435

B. Extraction Result of Biosurfactant and Critical Micelle Concentration (CMC) Determination

After biosurfactant produced and supernatant acquired, supernatant then extracted to obtain biosurfactant crude product. Biosurfactant dry weight acquired from 60% ammonium sulfate precipitation which obtained from *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61 and *Acinetobacter* sp. presented on Table 4. After crude product obtained, CMC from *Acinetobacter* sp. P2(1), *Pseudomonas putida* T1-8, *Bacillus subtilis* 3Kp and *Micrococcus* sp. L II 61 biosurfactant was determined.

Table 4. Dry weight of biosurfactant crude product extracted from 100 mL supernatant

Biosurfaktant Bacteria	Dry weight of crude product (g)	CMC (g/L)	Description
<i>Bacillus subtilis</i> 3Kp	6.111	16	≤CMC
<i>Acinetobacter</i> sp. P2(1)	4.494	5	≤CMC
<i>Pseudomonas putida</i> T1-8	3.782	3.75	≥CMC
<i>Micrococcus</i> sp. L II 61	4.017	4.73	≤CMC

Results was show that the four bacteria studied produced biosurfactant on different level. It had various emulsification activity and surface tension. From Table 4, it had known that the crude product of biosurfactant of *Acinetobacter* sp. P2(1) was as much as 5 g/L, while *Pseudomonas putida* T1-8 and *Micrococcus* sp. L II 61 was 3,75 g/L and 4,73 g/L respectively, and *Bacillus subtilis* 3Kp crude product weighted 16 g/L.

Previous study [12] stated on the same concentration with CMC (=CMC), biosurfactant would be formed more mycelles. This statement supported by high percentage of oil recovery effectiveness from *Acinetobacter* sp. P2(1) and *Pseudomonas putida* T1(8) on the same concentration with CMC (=CMC); respectively 48,62% and 13,81%. But those oil recovery effectiveness acquired from biosurfactant crude product application, while this study applied supernatant directly on oil recovery using sand pack column, where biosurfactant combined with lipase not always affected by the concentration (< CMC, = CMC, > CMC), towards oil recovery and percentage of crude oil solubility. Biosurfactant optimization might be conducted by increasing production or incubation duration, so the product acquired CMC (=CMC) or CMC (≥CMC).

Determination of CMC from the four biosurfactant crude products; *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61, and *Acinetobacter* sp. P2(1), purposed to understand the different characteristic of the four bacterias used. According to [9], surface tension, CMC, and emulsification stability were features and characteristics biosurfactant

product depending on substrate and bacteria type used. Efficiency and effectiveness of the four biosurfactants determined from CMC and surface tension [13].

A biosurfactant expressed effectively when it had 1-2000 mg/L [14]. Based on CMC and surface tension, biosurfactant crude product of *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus sp.* L II 61 and *Acinetobacter sp.* P2(1) categorized as less efficient, because the total CMC values >2000 mg/L. This was possibly caused by the form of biosurfactant that was crude product and still yet to be purified.

C. Supernatant Combination of Biosurfactant and Lipase Effectiveness Test in Oil Recovery using Sand Pack Column

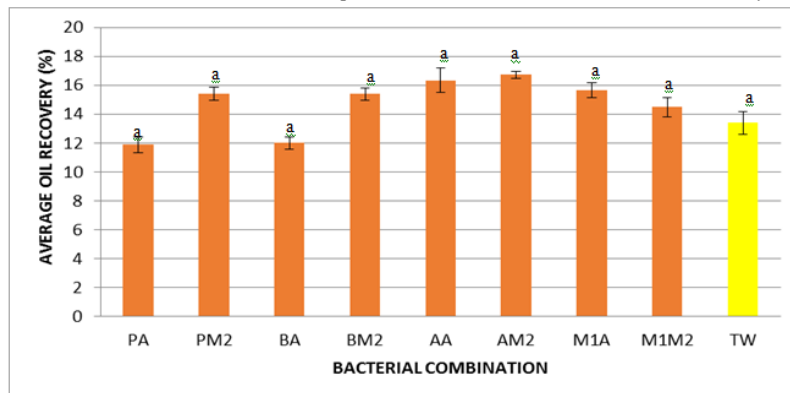


Fig. 1 Graphic of biosurfactant and crude enzyme combination effect on oil recovery percentage

Description:

- TW : Tween 20 (positive control)
- AA : *Acinetobacter sp.* P2(1) biosurfactant and *Actinobacillus sp.* lipase
- BA : *Bacillus subtilis* 3Kp biosurfactant and *Actinobacillus sp.* lipase
- PA : *Pseudomonas putida* T1(8) biosurfactant *Actinobacillus sp.* lipase
- M1A : *Micrococcus sp.* L II 61 biosurfactant and *Actinobacillus sp.* lipase
- AM2 : *Acinetobacter sp.* P2(1) biosurfactant and *Micrococcus sp.* L II 61 lipase
- BM2 : *Bacillus subtilis* 3Kp biosurfactant and *Micrococcus sp.* L II 61 lipase
- PM2 : *Pseudomonas putida* T1(8) biosurfactant and *Micrococcus sp.* L II 61 lipase
- M1M2 : *Micrococcus sp.* L II 61 biosurfactant and *Micrococcus sp.* L II 61 lipase

The result of oil recovery from various treatments conducted on the study presented on Fig 1. Oil recovery percentage from the eight biosurfactant and lipase combination analyzed statistically. Results of Kolmogorov-Smirnov test showed that data collected have normal distribution. Then data examined using Levene test and resulting in homogenous data type. With one-way ANOVA, data of the eight treatment combination of biosurfactant and lipase showed no significant effect on oil recovery using sand pack column method with significance degree (α) > 5 % (0,05).

Based on Fig 1, the eight combination treatment had not effected greatly on oil recovery using sand pack column method. The effectiveness in oil recovery of the eight combinations was all comparable to that of synthetic surfactant (Tween-20). Oil recovery using aquadest (negative control) reached to 1.62%, it was because a substance can be dissolved into solvent if both possess the same polarity; such as polar substance would be dissolved into polar solvent and not dissolved into non-polar solvent. Oil (lipids) was a non-polar compound, while aquadest was polar solvent. So the oil can't be dissolved into aquadest [15].

Results of oil recovery in positive control (Tween-20) obtained as much as 13.40%. The highest result from bacteria treatment acquired from combination of *Acinetobacter sp.* P2(1) biosurfactant and *Micrococcus sp.* L II 61 crude enzyme, with percentage of 16,73%.

Overall, all treatment conducted was able to enhance oil recovery, as evidenced by the resulting oil recovery percentage comparable to that of synthetic surfactant (Tween-20). The ability in removing oil caused by lipase crude enzyme works on oil (lipid)-water interfacial and degrades well oil (lipid) component on crude oil until it can be dissolved into water phase.

But the oil recovery of biosurfactant and lipase combination using sand pack column method did not result in high percentage compared to solubilization test using agitation method. From [16] study, *Bacillus subtilis* 3Kp biosurfactant combined with *Actinobacillus sp.* Were able to dissolve oil sludge as much as 40,671% on the same concentration of CMC, and from [7] study, *Acinetobacter sp.* P2(1) biosurfactant dissolve oil sludge by 40,93% on the same concentration of CMC.

Declines of oil recovery percentage on sand pack column method possibly caused by biosurfactant negative interaction factor with sand (soil) substrate. Sand or soil type used in the study affect greatly in releasing hydrocarbon bond with biosurfactant and crude oil. In addition, other factors also influence oil recovery percentage on sand pack column.

Although resulting oil recovery percentage was not quite high, but the effectiveness of *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus sp.* L II 61, *Acinetobacter sp.* P2(1) biosurfactant combined with *Actinobacillus sp.* and *Micrococcus sp.* L II 61 crude enzyme supernatant almost on the same level with synthetic surfactant (Tween-20). This

indicated that biosurfactant combination with lipase crude enzyme was potentially able to substitute synthetic surfactant usage in crude oil waste processing.

Results obtained in this study came from one times flushing or recover with 24 hours exposure time. Soil polluted with spilled oil will produce more optimum results by multiple flushing. But this theory was still yet to be proven; if oil recovery with multiple flushing would be resulted in increasing, decreasing, or constant percentage. It was interesting to reveal how oil recovery percentage resulted with application of multiple flushing on the four biosurfactant and two lipase types studied.

CONCLUSION

1. The four types of biosurfactant had different characters. *Bacillus subtilis* 3Kp was able to decline surface tension to 9.14 dyne/cm and emulsify oil as much as 18.85%. *Acinetobacter* sp. P2(1) was able to decrease surface tension up to 16.89 dyne/cm and emulsify oil by 24.94%. *Pseudomonas putida* T1(8) and *Micrococcus* sp. L II 61 was able to decline surface tension by 10.56 dyne/cm and 17.82 dyne/cm respectively, and also emulsified oil as much as 63.97% and 40.64%.
2. Both lipase types had different characters. *Micrococcus* sp. L II 61 and *Actinobacillus* sp. had lipolytic ability and possibly applied on oil recovery, respectively 11.137 (U/ml) and 7.553 (U/ml).
3. Combination of *Bacillus subtilis* 3Kp, *Pseudomonas putida* T1-8, *Micrococcus* sp. L II 61 biosurfactant with *Micrococcus* sp. L II 61 and *Acinetobacter* sp. P2(1) lipase had effected and resulted comparable to those of synthetic surfactant (Tween-20) on oil recovery using sand pack column method.

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